

## Article

# Waste Conversion into a Sweetener—Development of an Innovative Strategy for Erythritol Production by *Yarrowia lipolytica*

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Received: 17 July 2020; Accepted: 28 August 2020; Published: 1 September 2020



**Abstract:** The study proposed the innovative low-cost strategy for erythritol production by *Yarrowia lipolytica* through developing a simple medium based on industrial waste by-products and a natural method for culture broth purification. Results obtained proved that corn steep liquor might successfully replace traditional sources of nitrogen and other nutrients without compromising activities of the enzymes responsible for erythritol production and its production level. As a consequence, a production process was performed where *Y. lipolytica* A-6 was able to produce 108.0 g/L of erythritol, with a production rate of 1.04 g/Lh and a yield of 0.45 g/g of the medium containing exclusively 220 g/L of crude glycerol derived from biodiesel production and 40 g/L of corn steep liquor. Moreover, a comparable concentration of erythritol (108.1 g/L) was obtained when a part of crude glycerol was exchanged for the crude fraction of fatty acids in the two-steps process. Next, the collected post-fermentation broths were used in the culture with *Y. lipolytica* Wratislavia K1 for natural purification. The process resulted in a high increase of erythritol selectivity from 72% to 97% and in the production of 22.0 g/L of biomass with 40.4% protein content, which enables its use as an attractive animal feedstuff.

**Keywords:** erythritol; crude glycerol; fatty acids; *Yarrowia lipolytica*; purification

## 1. Introduction

In the last decade, modern trends in nutrition led to the increased consumption of erythritol. This polyol has many advantages, the primary of which includes low energy value (0.2 kcal/g), lack of bad aftertaste, not affecting insulin level in the blood, and very good tolerance in the human digestive system, which makes it one of the best sugar substitutes [1].

The market demand for erythritol has meant that, in recent years, many works have been devoted to it and reported the conditions of its biosynthesis [2]. The possibility of production cost reduction was investigated by, e.g., screening the best-producing strains and their genetic modifications [3,4], the use of various culture systems [5–7], optimization of culture conditions [8–10], and the use of various substrates [8,11–14]. Among the various substrates tested, the use of the media based on industrial waste by-products seems to be the most prospective for biotechnological processes, by being both cost-effective and environmentally-friendly.

*Yarrowia lipolytica* yeast is one of the most extensively studied organisms in respect of erythritol production. The environmental conditions, as well as the biochemical pathways of this polyol biosynthesis, have been widely described [2,15,16]. This non-conventional yeast has low growth requirements, is able to assimilate a wide spectrum of substrates, and has fair resistance to impurities

and changes in the composition of the culture medium [17]. Moreover, it might be successfully applied to the continuous and semi-continuous systems [5,18], and is characterized by a good growth at a low pH of the environment, which minimizes the risk of bacterial contamination of the process [19]. Different natural and industrial-waste compounds were used as culture media components in the studies addressing erythritol biosynthesis by *Y. lipolytica*, these being: crude glycerol [16,20]; raw molasses [6]; olive oil, soybean oil, rapeseed oil, and waste cooking oil [21,22]; fungi-pretreated soybean residue and buckwheat husk [23,24]; corn steep liquor [7,21]; or inulin [25]. However, up to date, studies have employed substrates that required specific pretreatment or supplementation with complex nutrients, which, despite the non-expensive substrate application, might prove cost-ineffective.

The objective of the presented study was to develop a simple medium based on industrial waste by-products and a natural method for the purification of the obtained culture broth, which altogether aimed at developing a low-cost strategy for effective erythritol production by *Y. lipolytica* yeast.

## 2. Materials and Methods

### 2.1. Microorganisms

The wild strains A-3, A-6, and A-311, isolated in 1974 from soil, and an acetate-negative mutant Wratislavia K1 of *Yarrowia lipolytica* used in this study originated from the yeast culture collection of the Department of Biotechnology and Food Microbiology, Wrocław University of Environmental and Life Sciences in Poland [26]. The strains were stored on YM slants at 4 °C.

### 2.2. Substrates

Carbon and energy sources in the media applied included: pure glycerol (98%), crude glycerol from methyl ester production (SG BODDINS GmbH; Germany) containing 86% wt/wt of glycerol, 6.5% wt/wt of NaCl, and methanol-below 0.2%, and the crude fraction of fatty acids (CFFA) remained after the ethanolic esterification of fatty acids contained in rapeseed oil (pilot installation at the University of Wrocław; Poland).

### 2.3. Media

Two types of growth medium were used for seed culture preparation: glycerol-based growth medium (GGM) for the inoculation of mineral shake-flasks medium (MS-FM) and bioreactor media (mineral production medium—MPM; low cost production medium—LPM; purification process medium—PPM), and fatty acid crude fraction-based growth medium (FGM) to inoculate bioreactor cultures (fatty acid crude fraction based production medium—FPM and waste substrates based production medium—WPM) in which the production medium contained CFFA. The growth media (GGM, FGM) consisted of (g/L): CFFA, 20.0, or pure glycerol, 50.0, as the substrates (in FGM and GGM medium, respectively), yeast extract, 3.0, malt extract, 3.0, and bactopectone, 5.0, dissolved in distilled water.

Erythritol biosynthesis in the shake-flasks experiment was performed in the mineral medium (MS-FM) that consisted of (g/L): Pure glycerol, 100.0,  $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ , 1.0,  $\text{KH}_2\text{PO}_4$ , 0.2, yeast extract, 1.0, and corn steep liquor (CSL) (Sigma) 4.0–40.0,  $\text{CaCO}_3$ , 3.0, dissolved in distilled water, pH 3.0.

Different media (FPM, MPM, LPM, WPM, PPM) were used in erythritol production in bioreactor cultures. The fatty acid fraction-based production medium (FPM) contained (g/L): CFFA, 20.0, pure glycerol, 200.0,  $(\text{NH}_4)_2\text{SO}_4$  4.6,  $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ , 1.0,  $\text{KH}_2\text{PO}_4$ , 0.25, yeast extract, 1.0, and NaCl, 0.0/25.0, dissolved in tap water, pH 3.0. The composition of the mineral production medium (MPM) was as follows (g/L): pure or crude glycerol, 150.0/220.0, CSL, 24.0/40.0,  $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ , 1.0,  $\text{KH}_2\text{PO}_4$ , 0.2, yeast extract, 1.0, and NaCl, 0/25.0/32.5 in 1 L of tap water, pH 3.0. The low-cost production medium (LPM) contained (g/L): pure or crude glycerol, 220.0, and CSL dissolved in 1 L of tap water, pH 3.0. In turn, the waste-substrate based production medium (WPM) was prepared with the use of (g/L): CFFA, 20.0, CSL, 40.0, NaCl, 25.0, and pure/crude glycerol, 150.0/200.0, dissolved in 1 L of

tap water, pH 3.0. To obtain 24.0 or 40.0 g/L of CSL, 48.0 or 80.0 g/L of 50% CSL (Sigma-Aldrich) was introduced to the medium. To prepare the purification process medium (PPM), the culture broth after earlier performed erythritol biosynthesis processes was collected, separated from the biomass by filtration ( $\phi$  0.45  $\mu$ m), sterilized (121 °C, 20 min), and stored at 2 °C. Finally, the previously prepared liquids were supplemented with (g/L):  $(\text{NH}_4)_2\text{SO}_4$  4.6,  $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ , 1.0,  $\text{KH}_2\text{PO}_4$ , 0.25, and yeast extract, 1.0, pH 3.0/4.5. In the processes with FPM and WPM media, an appropriate glycerol portion was introduced to the culture after 24 h of cultivation, whereas, in the case of the MPM and LPM media, glycerol was present from the beginning of the process. All the media were sterilized at 121 °C for 20 min.

#### 2.4. Culture Conditions

The growth cultures (GGM, FGM) were carried out in 0.3-L flasks containing 0.1 L of the growth medium on a rotary-shaker (CERTOMAT IS, Sartorius Stedim Biotech GmbH) at 29.5 °C and 140 rpm for 3 days. In order to inoculate the media for the shake-flask and bioreactor experiments, 3 mL and 200 mL of the growth culture were used, respectively. The shake-flasks experiment was conducted for 7 days in 0.3-L flasks containing 0.03 L of the appropriate production medium (MS-FM), inoculated with 1 mL of seed culture, in the same conditions as described above. The samples were taken for analysis at the end of the experiment. Bioreactor cultures were carried out in a 5-L stirred-tank reactor (Biostat B Plus, Sartorius, Germany) with a working volume of 2 L, at 30 °C, the aeration rate fixed at 0.6 v/v/min, and the stirrer speed adjusted to 800 rpm. The pH of 3.0 was maintained automatically by the addition of a 20% (w/v) NaOH solution. All the bioreactor cultures were cultivated until glycerol was completely consumed. Samples were withdrawn 2–3 times per day. Results are presented as mean values of three biological replicates for the shake-flasks experiment and two biological replicates for the cultures conducted in bioreactor.

#### 2.5. Analytical Methods

Biomass content of the samples was determined gravimetrically after drying at 105 °C. Concentrations of glycerol, erythritol, mannitol, arabitol, citric acid, and  $\alpha$ -ketoglutaric acid were determined with the HPLC method [14]. The osmotic pressure was measured with a Marcel OS 3000 osmometer (Marcel, Poland).

#### 2.6. Enzyme Assays

After 24 h of cultivation, the samples (0.2 L) were withdrawn from the bioreactors. Extracts were prepared, protein content was determined, and enzyme activities were analyzed according to the methodology described by Tomaszewska et al. [16]. The activity of the following enzymes was determined in the supernatant: glycerol kinase (GK) (EC 2.7.1.30); glycerol-3-phosphate dehydrogenase (GPDH) (EC 1.1.1.8); transketolase (TK) (EC 2.2.1.1); and erythrose reductase (ER) (EC 1.1.1.21). One unit (U) of enzyme activity was defined as 1  $\mu$ mol of NADH/NADPH consumed or produced per 1 min ( $\lambda$  = 340 nm). The enzymatic activity was expressed as U per mg of protein (U/mg of protein).

#### 2.7. Statistical Analysis

To detect significant differences in the data, one-way analysis of variance was performed using Statistica 12.5 software (StatSoft, Tulsa, OK, USA). Homogeneous groups were determined using the Duncan's test ( $p \leq 0.05$ ).

### 3. Results

#### 3.1. The Effect of Crude Fatty Acid Fraction Application

Three natural strains, A-3, A-6, and A-311, of *Y. lipolytica* were examined for erythritol biosynthesis in the two-stage process (Table 1) in the FPM medium. In the first, growth stage only, CFFA was

present in the medium as the source of carbon and energy. Next, after 24 h of cultivation, the erythritol production phase was initiated by glycerol addition to the culture. All the strains under investigation were able to assimilate both substrates; however, the level of biomass in the stationary phase depended on the strain and was the highest for the A-6 strain (about 34.4 g/L). The process was run until complete depletion of glycerol, which lasted from 92.0 h in the case of strain A-6 to 169.5 h when strain A-311 was applied, resulting in erythritol production of 66.0 to 86.3 g/L, respectively. Simultaneously, by-production of mannitol exceeded 21.0 g/L for strain A-3 and 40.0 g/L for the two other strains under investigation. Additionally, some culture media were supplemented with NaCl in two variants: salt applied as a component of initial medium or by its simultaneous addition with glycerol portion after 24 h of the cultivation process. The use of NaCl increased the osmotic pressure of the culture media (from 0.2 up to 3.4 Osm/kg) and allowed for higher erythritol production (up to 125.3 g/L) and significant improvement of the production yield due to reduction of mannitol by-production. The best parameters of erythritol production, i.e., productivity of 1.19 g/Lh and specific production rate of 0.035 g/gh, were obtained for the strain A-6 in the culture in which NaCl was present in the medium from the beginning of the process.

**Table 1.** Comparison of erythritol production process by different strains of *Y. lipolytica* on media containing crude fraction of fatty acids (CFFA).

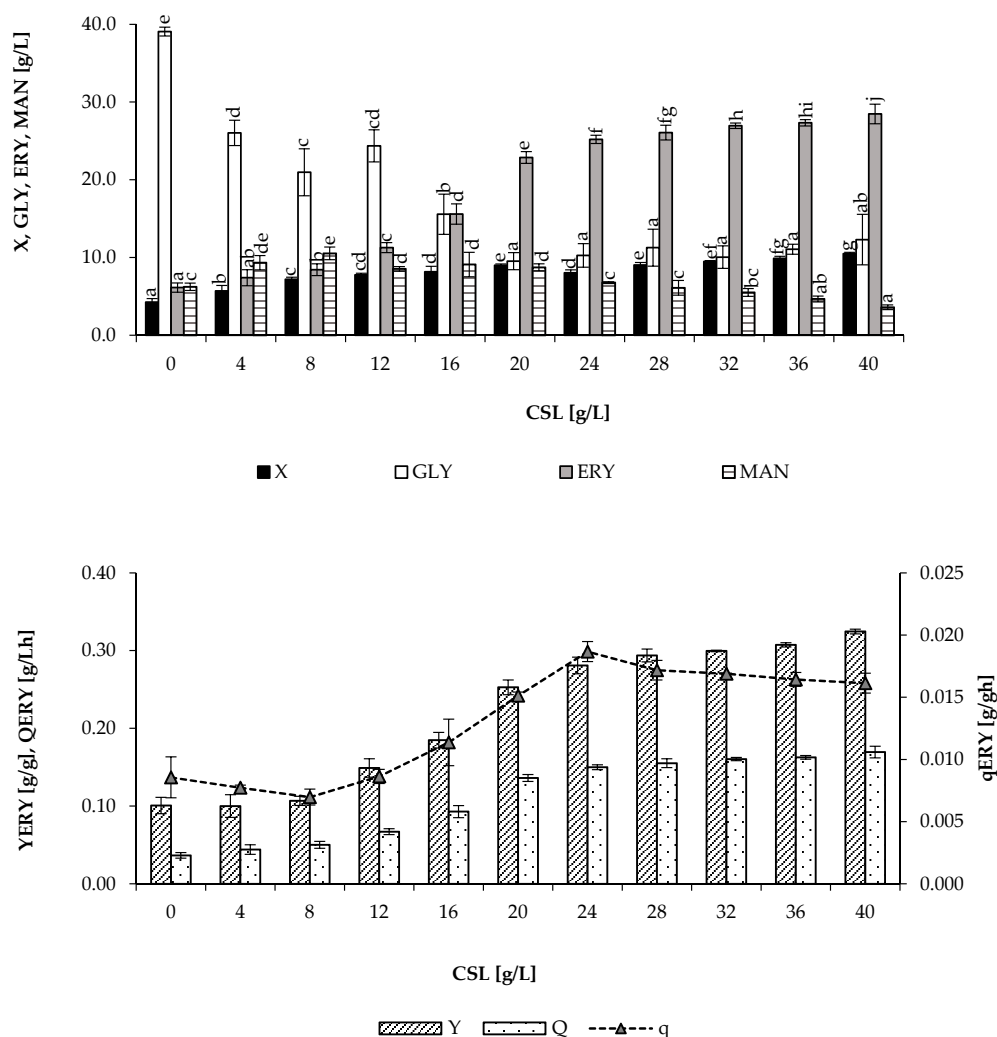
	NaCl	Time *	X	ERY	MAN	Y <sub>ERY</sub>	Q <sub>ERY</sub> **	q <sub>ERY</sub> **	Osmotic Pressure ***
[g/L]	Supplementation Method	[h]		[g/L]		[g/g]	[g/Lh]	[g/gh]	[Osm/kg]
Y. lipolytica A-3									
-	-	125.0 ± 4.1 <sup>b,c</sup>	28.8 ± 2.3 <sup>c,d</sup>	72.5 ± 3.7 <sup>a,b</sup>	21.9 ± 3.9 <sup>c</sup>	0.37	0.72	0.025	0.2→2.6
25	with glycerol addition	135.5 ± 5.5 <sup>c</sup>	27.3 ± 1.2 <sup>c</sup>	89.7 ± 5.2 <sup>c</sup>	12.1 ± 4.4 <sup>b</sup>	0.42	0.81	0.030	0.2→3.4
25	in the initial medium	115.5 ± 3.3 <sup>b</sup>	27.1 ± 2.1 <sup>c</sup>	87.1 ± 4.4 <sup>c</sup>	5.3 ± 2.9 <sup>a</sup>	0.43	0.96	0.035	1.1→3.3
Y. lipolytica A-6									
-	-	92.0 ± 4.0 <sup>a</sup>	32.3 ± 4.5 <sup>d,e</sup>	66.0 ± 4.9 <sup>a</sup>	43.0 ± 6.2 <sup>d</sup>	0.34	0.97	0.030	0.2→2.3
25	with glycerol addition	125.5 ± 6.0 <sup>b,c</sup>	34.3 ± 3.8 <sup>e</sup>	75.6 ± 3.5 <sup>b</sup>	15.1 ± 1.8 <sup>b,c</sup>	0.37	0.74	0.022	0.2→3.2
25	in the initial medium	99.5 ± 2.6 <sup>a</sup>	34.4 ± 1.9 <sup>e</sup>	89.5 ± 2.4 <sup>c</sup>	16.0 ± 3.0 <sup>b,c</sup>	0.43	1.19	0.035	1.1→3.1
Y. lipolytica A-311									
-	-	169.5 ± 6.8 <sup>d</sup>	21.2 ± 1.6 <sup>a,b</sup>	86.3 ± 5.1 <sup>c</sup>	41.0 ± 5.1 <sup>d</sup>	0.43	0.59	0.028	0.2→2.1
25	with glycerol addition	247.0 ± 10.1 <sup>f</sup>	25.5 ± 3.5 <sup>b,c</sup>	125.3 ± 7.0 <sup>e</sup>	21.4 ± 3.3 <sup>c</sup>	0.58	0.56	0.022	0.2→3.1
25	in the initial medium	191.0 ± 7.6 <sup>e</sup>	20.2 ± 1.5 <sup>a</sup>	107.3 ± 4.3 <sup>d</sup>	16.4 ± 2.3 <sup>b,c</sup>	0.52	0.65	0.032	1.1→3.3

Conditions: fraction-based production medium (FPM) with CFFA 20 g/L and pure glycerol 200 g/L (addition after 24-h of cultivation). Means in the same column marked with different letters (a, b, ...) are significantly different;  $p \leq 0.05$ . \* Total time of the process; \*\* Calculated for glycerol conversion in respect of the time when it was introduced to the culture broth; \*\*\* Initial osmotic pressure of the culture broth and its change resulting from glycerol addition. X—biomass, ERY—erythritol, MAN—mannitol, Y<sub>ERY</sub>—erythritol production yield, Q<sub>ERY</sub>—volumetric erythritol production rate, q<sub>ERY</sub>—specific erythritol production rate.

### 3.2. The Effect of CSL Application

The impact of CLS on erythritol biosynthesis from glycerol by the A-6 strain of *Y. lipolytica* was examined in the shake-flask (Figure 1) and bioreactor cultures (Table 2), respectively in the MS-FM and MPM media. In the shake-flask experiment, the increase of CSL concentration from 0.0 to 40 g/L resulted in the increase of biomass level from 4.3 to 10.5 g/L and erythritol concentration from 6.1 to 28.5 g/L (Figure 1). With the enhanced erythritol biosynthesis, the volumetric and specific production rates, as well as the yield of erythritol production, improved up to 0.17 g/Lh, 0.016 g/gh, and 0.32 g/g, respectively. However, the strongest stimulatory effect was observed when the CLS concentration was increased to 24.0 g/L, whereas its further increase to 40.0 g/L had no longer such a strong impact on erythritol production. Therefore, both variants were chosen for comparison with the bioreactor

production cultures performed in the MPM medium that contained 150 g/L of glycerol and 25 g/L of NaCl. The process with the higher dose of CSL was almost half as much (147 h), and the yeast biomass level was twice as much (25.4 g/L) in comparison to the culture with 24.0 g/L of CSL (Table 2). Moreover, the application of 40.0 g/L of CSL enhanced erythritol biosynthesis, causing erythritol production at 83.0 g/L with a yield of 0.56 g/g and productivity of 1.08 g/Lh.



**Figure 1.** Comparison of erythritol production by *Y. lipolytica* A-6 in the shake-flasks experiment, depending on corn steep liquor (CSL) concentration in the culture medium. Conditions: (mineral shake-flasks medium (MS-FM)). Means of the same parameter marked with different letters (a, b, ...) are significantly different;  $p \leq 0.05$ . X—biomass, GLY—glycerol, ERY—erythritol, MAN—mannitol,  $Y_{ERY}$ —erythritol production yield,  $Q_{ERY}$ —volumetric erythritol production rate,  $q_{ERY}$ —specific erythritol production rate.

**Table 2.** Comparison of erythritol biosynthesis by *Y. lipolytica* A-6 depending on CSL concentration in the mineral medium with NaCl.

CSL [g/L]	Time [h]	X	ERY [g/L]	MAN	$Y_{ERY}$ [g/g]	$Q_{ERY}$ [g/Lh]	$q_{ERY}$ [g/g/h]
24	$147 \pm 4.7^a$	$11.5 \pm 1.8^a$	$75 \pm 3.6^a$	$4.2 \pm 2.9^a$	0.52	0.51	0.040
40	$77 \pm 3.5^b$	$25.4 \pm 2.6^b$	$83 \pm 1.7^b$	$2.2 \pm 1.5^a$	0.56	1.08	0.042

Conditions: mineral production medium (MPM) with pure glycerol 150 g/L (in the initial medium) and 25 g/L of NaCl. Means in the same column marked with different letters (a, b, ...) are significantly different;  $p \leq 0.05$ .

### 3.3. Comparison of Factors Causing the Increase of the Osmotic Pressure

Biosynthesis of erythritol from glycerol by the A-6 strain of *Y. lipolytica* was compared in terms of an increased osmotic pressure caused by an increased substrate concentration or NaCl presence in the MPM medium with 40.0 g/L CSL. In the case of both factors, the increase of the osmotic pressure resulted in the significant shortening of the process and increased erythritol production (Table 3). The yield and productivity of erythritol production were improved; however, the values of these parameters were higher in the case of NaCl application and reached 0.49 g/g and 1.0 g/Lh, respectively. The analysis of enzyme activities showed that the higher dose of the substrate stimulated the activity of glycerol kinase (Table 3). The stimulatory effect of glycerol and NaCl was similar in the case of transketolase activity, which reached 0.064 and 0.069 U/mg, respectively. However, it was noted that erythrose reductase activity (0.120 U/mg) was enhanced to a greater extent by salt presence in the medium.

**Table 3.** Biosynthesis parameters and enzymatic activity during erythritol production by *Y. lipolytica* A-6 depending on the factor causing the increase of the osmotic pressure in the medium.

Medium	Osmotic Pressure	Time	X	ERY	MAN	Y <sub>ERY</sub>	Q <sub>ERY</sub>	q <sub>ERY</sub>	GK	GPDH	TK	ER
	[Osm/kg]	[h]		[g/L]		[g/g]	[g/Lh]	[g/gh]			[U/mg]	
without NaCl 150 g/L of glycerol	2.1	108 ± 4.8 <sup>a</sup>	28.1 ± 3.0 <sup>a</sup>	62.2 ± 2.8 <sup>a</sup>	22.8 ± 3.1 <sup>a</sup>	0.40	0.58	0.020	0.021 ± 0.002 <sup>a,b</sup>	0.016 ± 0.001 <sup>b</sup>	0.051 ± 0.005 <sup>a</sup>	0.060 ± 0.006 <sup>a</sup>
without NaCl 220 g/L of glycerol	3.1	99 ± 3.5 <sup>b</sup>	31.0 ± 1.8 <sup>a</sup>	93.0 ± 4.6 <sup>b</sup>	20.1 ± 1.9 <sup>a</sup>	0.42	0.95	0.030	0.024 ± 0.001 <sup>b</sup>	0.003 ± 0.000 <sup>a</sup>	0.064 ± 0.004 <sup>b</sup>	0.075 ± 0.004 <sup>b</sup>
32.5 g/L NaCl 150 g/L of glycerol	3.2	74 ± 3.3 <sup>c</sup>	27.0 ± 1.7 <sup>a</sup>	74.4 ± 5.4 <sup>c</sup>	2.4 ± 2.1 <sup>b</sup>	0.49	1.00	0.037	0.020 ± 0.002 <sup>a</sup>	0.017 ± 0.001 <sup>b</sup>	0.069 ± 0.001 <sup>b</sup>	0.120 ± 0.009 <sup>c</sup>

Conditions: MPM with pure glycerol (in the initial medium) and 40 g/L of CSL. Means in the same column marked with different letters (a, b, ...) are significantly different;  $p \leq 0.05$ . GK—glycerol kinase, GPDH—glycerol-3-phosphate dehydrogenase, TK—transketolase, ER—erythrose reductase.

### 3.4. Comparison of Mineral and Low-Cost Medium

Pure and crude glycerol (220 g/L) were used in the mineral (MPM containing 40.0 g/L of CSL and 25.0 g/L of NaCl) and low-cost (LPM) media used for erythritol biosynthesis by *Y. lipolytica* A-6 (Table 4). When pure substrate was used, about 93.0 g/L of erythritol was produced in both the mineral and the low-cost medium, which corresponded to the yield of about 0.40 g/g. Elimination of mineral components in the low-cost medium led to prolonged biosynthesis time (159 h) and a twice lower biomass level (16.4 g/L), which resulted also in the lower productivity and specific production rate, in comparison to the culture with the mineral medium. The use of crude glycerol resulted in very similar results of erythritol biosynthesis obtained in mineral and low-cost media. In both media, within about 100 h, yeast were able to produce about 110 g/L of erythritol with the productivity of about 1.1 g/Lh and specific production rate of 0.040 g/gh.

**Table 4.** Comparison of erythritol production from pure and crude glycerol by A-6 strain of *Y. lipolytica* in mineral and low-cost medium.

Medium	Glycerol	Time	X	ERY	MAN	Y <sub>ERY</sub>	Q <sub>ERY</sub>	q <sub>ERY</sub>
		[h]		[g/L]		[g/g]	[g/Lh]	[g/gh]
Mineral (MPM)	Pure	99 ± 4.3 <sup>a</sup>	31.8 ± 3.4 <sup>c</sup>	93.0 ± 4.2 <sup>a</sup>	20.2 ± 2.6 <sup>a</sup>	0.42	0.95	0.030
Low-cost (LPM)	Pure	159 ± 5.1 <sup>b</sup>	16.4 ± 2.2 <sup>a</sup>	94.0 ± 3.5 <sup>a</sup>	17.0 ± 2.4 <sup>a</sup>	0.40	0.59	0.036
Mineral (MPM)	Crude	100 ± 4.1 <sup>a</sup>	28.8 ± 1.7 <sup>b,c</sup>	111.0 ± 6.3 <sup>b</sup>	6.1 ± 3.1 <sup>b</sup>	0.50	1.11	0.039
Low-cost (LPM)	Crude	104 ± 3.3 <sup>a</sup>	25.4 ± 2.3 <sup>b</sup>	108.0 ± 2.1 <sup>b</sup>	8.1 ± 1.2 <sup>b</sup>	0.45	1.04	0.041

Conditions: MPM and low-cost production medium (LPM) with 220 g/L of pure/crude glycerol (in the initial medium) and 40 g/L of CSL; MPM medium additionally supplemented with 25 g/L of NaCl. Means in the same column marked with different letters (a, b, ...) are significantly different;  $p \leq 0.05$ .



### 3.5. Development of Waste-Substrate Based Medium

The WPM medium, containing only natural and waste substrates (see Materials and Methods Section), was proposed based on the previous experiments. The process of erythritol biosynthesis with the A-6 strain of *Y. lipolytica* was performed in two stages, according to the strategy carried out earlier in the process with the FPM medium: Biomass was produced when only CFFA was present in the culture medium, and erythritol biosynthesis was initiated after about 24 h by pure or crude glycerol introduction to the culture broth. In the culture with 150 g/L of crude glycerol, biomass concentration reached 20.3 g/L and yeast were able to produce 63.5 g/L of erythritol (Table 5) with a yield of 0.42 g/g and a productivity of 0.42 g/Lh. The application of the higher concentration (200 g/L) of the substrate resulted in a process extension to 208.5 h; however, the biomass level and parameters of erythritol biosynthesis increased. In the culture with pure and crude glycerol (200 g/L), erythritol concentration reached 110.3 and 108.1 g/L, which corresponded to the productivity of 0.67 and 0.57 g/Lh, respectively.

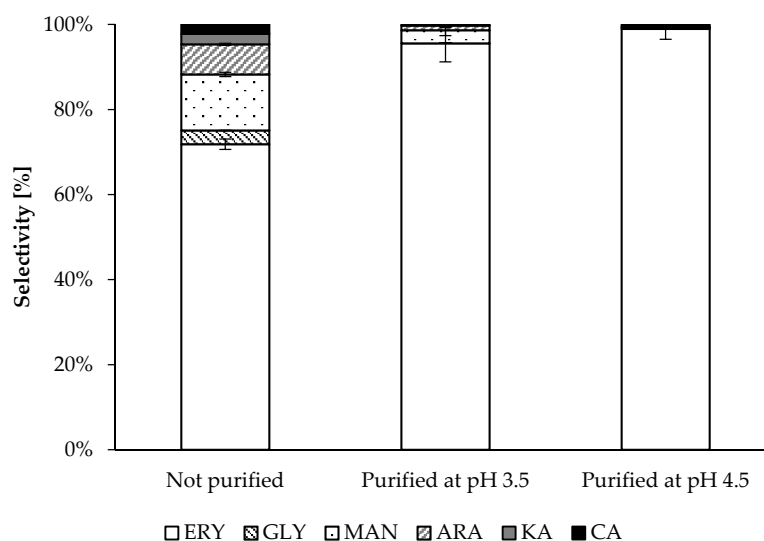
**Table 5.** Erythritol biosynthesis on waste substrate medium by *Y. lipolytica* A-6.

Glycerol	Time *	X	ERY	MAN	Y <sub>ERY</sub>	Q <sub>ERY</sub> **	q <sub>ERY</sub> **
	[h]		[g/L]		[g/g]	[g/Lh]	[g/gh]
Pure 200 g/L	187.0 ± 5.5 <sup>b</sup>	28.1 ± 2.7 <sup>b</sup>	110.3 ± 6.6 <sup>b</sup>	14.8 ± 3.0 <sup>a</sup>	0.51	0.67	0.024
Crude 200 g/L	208.5 ± 6.1 <sup>c</sup>	26.4 ± 2.9 <sup>b</sup>	108.1 ± 3.3 <sup>b</sup>	10.8 ± 1.9 <sup>a</sup>	0.47	0.57	0.022
Crude 150 g/L	172.0 ± 4.6 <sup>a</sup>	20.3 ± 1.7 <sup>a</sup>	63.5 ± 4.4 <sup>a</sup>	12.4 ± 1.8 <sup>a</sup>	0.42	0.42	0.021

Means in the same column marked with different letters (a, b, ...) are significantly different;  $p \leq 0.05$ . \* Total time of the process; \*\* Calculated for glycerol conversion in respect of the time when it was introduced to the culture broth. Conditions: waste-substrate based production medium (WPM) with 20 g/L of CFFA and pure/crude glycerol (addition after 24 h of cultivation).

### 3.6. Natural Purification Process

The purification process, which was intended to increase the selectivity of erythritol in the post-culture broth, was performed in the PPM medium at pH 3.0 and 4.5 with the use of *Y. lipolytica* Wratislavia K1 strain. The PPM medium initially contained 84.4 g/L of erythritol, 15.5 g/L of mannitol, 8.3 g/L of arabinol, and citric and ketoglutaric acids in the concentration of 2.6 and 2.9 g/L, respectively (Figure 2). Hence, the selectivity was 72%. At pH 3.0, after 64 h cultivation, the selectivity of the broth increased to 99% and 14.6 g/L of the biomass with 38.2% of protein content being produced. The increase of the pH value to 4.5 resulted in the purification process shortening to 49 h. After the process, the selectivity reached 96%, whereas biomass concentration was 14.6 g/L and its protein content was 40.4%.



**Figure 2.** Comparison of culture broths composition before and after the natural purification process performed with the use of *Y. lipolytica* Wratislavia K1. Conditions: purification process medium (PPM). ERY—erythritol, GLY—glycerol, MAN—mannitol, ARA—arabitol, KA— $\alpha$ -ketoglutaric acid, CA—citric acid.

#### 4. Discussion

The cost of the fermentation medium is a critical factor for the industrial biosynthesis process. Therefore, the cost-effectiveness of biosynthesis significantly depends on the cost of the substrate and the other components of the production medium. The natural feature of *Y. lipolytica* yeast is the capability for assimilation of hydrophilic and hydrophobic substrates, which allows the use of a wider range of substrates, including low-cost renewable resources or crude industrial wastes [21]. However, the biochemical pathways of utilization of these two types of substrates are different, which makes their use dependent on the specific direction of biosynthesis and the desired product to be obtained. In the first step of the presented study, two types of substrates were applied: glycerol and crude fatty acid fraction. *Y. lipolytica* yeast use the phosphorylation pathway, in which glycerol kinase (GK) catalyzes glycerol phosphorylation to glycerol-3-phosphate, which is subsequently converted via dehydrogenation by glycerol-3-phosphate dehydrogenase (GPDH) to dihydroxyacetone-phosphate that might be integrated into different pathways, resulting in biomass, polyols, lipids, citric, pyruvic, or  $\alpha$ -ketoglutaric acids formation [27,28]. In turn, fatty acids are directly metabolized into acetyl-CoA throughout  $\beta$ -oxidation. Acetyl-CoA is next incorporated to the citric acid cycle and might be easily converted to different acids of the Krebs cycle or used for biomass growth [29,30]. Considering different biochemical routes of utilization, the process of erythritol biosynthesis (Table 1) was divided into two steps: the first stage of biomass growth on the CFFA as the sole substrate in the medium, and the second stage of erythritol formation induced by glycerol introduction to the culture broth (with simultaneous increase of osmotic pressure of the culture medium). Different abilities to utilize and to grow on the CFFA were observed for the strains under investigation, resulting in different levels of biomass produced. Erythritol production was accompanied by the synthesis of a relatively high amount of mannitol (21.9–43.0 g/L). Earlier studies have shown that the by-production of mannitol might be reduced by increasing the osmotic pressure of the medium [31,32]. Therefore, a higher osmotic pressure was induced by the addition of 25 g/L of NaCl in two variants: at the beginning and after 24-h of the cultivation process (Table 1). The method of NaCl incorporation to the culture had no significant impact on biomass level but enhanced erythritol production to 125.3 g/L and reduced mannitol by-production to 75%. Yang et al. [32] reported that the increase of osmotic pressure to 3.89 Osm/kg in the cultures with *Y. lipolytica* grown on 200 g/L of glycerol, achieved by initial medium supplementation with 20 g/L of NaCl, decreased biomass production (from 16.0 to



11.9 g/L), increased erythritol production from 55.7 to 86.7 g/L, and decreased mannitol concentration from 50.4 to 27.7 g/L. In the present study, in the cultures with the rapid increase of osmotic pressure resulting from simultaneous introduction of NaCl and a high concentration of glycerol, the total time of the biosynthesis was prolonged, which is in agreement with earlier reports [31]. However, in the presented work, a very desirable effect of process shortening was obtained in the culture with NaCl, which improved also the erythritol productivity parameters in the cultures in which yeast cells adapted to the conditions of increased osmotic pressure from the beginning of the cultivation. The high osmotic pressure (2.76 Osm/L), induced by the addition of 80 g/L of NaCl, was reported as necessary for effective erythritol biosynthesis by *Y. lipolytica* from a waste cooking oil and allowed the production of 21.8 g/L of erythritol with the yield 0.80 g/g in the shake-flasks experiment and 22.1 g/L of the polyol with the yield of 0.74 g/g in the bioreactor culture [21,22].

Apart from the substrate, another necessary component of the medium is the nitrogen source. Yeast extract and peptone are usually used in biosynthesis processes as the organic sources of nitrogen; however, their use should be avoided due to their high cost. CSL was reported as an economic and effective substitute for the traditionally used nitrogen sources, e.g., in the production of bioemulsifier, biodiesel, erythritol, and citric and succinic acids by different microorganisms, including *Y. lipolytica* [7,21,33–35]. This organic component is a by-product of the corn steeping process of the maize-starch industry, and is rich in amino acids, vitamins, and polypeptides. In the presented study, the effect of CSL concentration on erythritol production was investigated in shake-flask (Figure 1) and bioreactor batch cultures (Table 2). In the shake-flask experiment, the increasing concentration of CSL enhanced erythritol biosynthesis, but the impact of CSL concentrations above 24 g/L was very small. However, the process performed in the bioreactor proved that the application of 40.0 g/L of CSL resulted in a significant improvement, especially in the parameters of erythritol production, as the process shortened to 77 h, which was half in comparison to the process with 24.0 g/L of CSL. Generally, the enhanced biomass growth results in decreased formation of process metabolites. Therefore, noteworthy is the fact that, in the presented experiment (Figure 1, Table 2), the increased concentration of CSL stimulated biomass growth; however, the yield of erythritol production increased simultaneously. The same observation was reported earlier for erythritol biosynthesis by *Yarrowia lipolytica* Wratislavia K1 [7] and was supposed to be the effect of vitamins and other component of the CSL, as it is a complex substance of natural origin. Under similar conditions (40.0 g/L of CSL and 150 g/L of glycerol), Wratislavia K1 strain was able to produce 61.0 g/L of erythritol with the productivity of 0.85 g/Lh and the yield of 0.38 g/g, which was significantly lower than in the case of the A-6 strain of *Y. lipolytica* in the present study.

NaCl is a functional component of the medium, added to increase the osmotic pressure and stimulate erythritol biosynthesis in cells and is not consumed by the yeast, therefore it remains in the broth after the end of the culture and has to be removed at the steps of product purification. However, the osmotic pressure increase might also be triggered by increasing the concentration of other medium components, including the substrate. Therefore, we compared the effect of osmotic pressure regulation by the increase of substrate concentration and NaCl in erythritol biosynthesis by *Y. lipolytica* (Table 3). The initial osmotic pressure of the medium was increased to the level of about 3.0 Osm/kg by the simultaneous application of salt (32.5 g/L) and glycerol (150 g/L) or only by the substrate (220 g/L). In comparison to the process with NaCl, in the culture conducted without salt the erythritol productivity remained at a high level of 0.95 g/Lh, and higher availability of the substrate resulted in the production of 93.0 g/L of erythritol. The use of CSL did not have any negative impact on the activities of glycerol kinase, glycerol-3-phosphate dehydrogenase, or transketolase (Table 3), compared to the culture with *Y. lipolytica* K1 grown on media with  $\text{NH}_4\text{Cl}$  being a mineral source of nitrogen [16]. The higher glycerol concentration slightly stimulated the activity of glycerol kinase, the enzyme responsible for the first step of glycerol consumption. Moreover, the increased osmotic pressure stimulated by glycerol and by the presence of both glycerol and NaCl enhanced the activities

of transketolase and erythrose reductase, which correlated with the increased amount of erythritol produced by the yeast in these cultures and is in agreement with earlier studies [16,36].

The other contrivance for cost reduction is to eliminate the unnecessary ingredients of the medium. As mentioned above, CSL is a complex substance of natural origin, and might be used, not only as a source of nitrogen, but also as a source of vitamins and trace elements in a fermentation medium [37]. Thus, in the next experiment we have verified the possibility of eliminating mineral components of the medium and performed erythritol biosynthesis in the simple medium containing only the substrate and CSL (Table 4). When pure glycerol was applied, the production of erythritol and by-formation of mannitol were similar in the medium with and without the minerals; however, the elimination of minerals resulted in prolonged biosynthesis time, which was not a desired effect. This was not observed when crude glycerol was used as the substrate, as there were no significant differences between the culture with the rich medium and the medium without minerals. It is well-known that crude glycerol contains impurities of natural origin, e.g., vitamins and trace elements [38]. Therefore, it might be concluded that the combined application of CSL and crude glycerol in the performed experiment provided a sufficient level of nutrients and minerals for erythritol biosynthesis.

In the next step, erythritol production was also performed in the simple medium with CSL and without mineral supplementation; however, an amount of glycerol (20 g/L) was replaced by the equal portion of the CFFA. The results obtained showed that erythritol biosynthesis by *Y. lipolytica* was possible in the medium composed only from waste products such as CSL, CFFA, and crude glycerol, which offers a highly cost-effective possibility for the commercial process. Concentration of biomass (26.4 g/L) and erythritol (108.1 g/L) obtained in the two-steps biosynthesis process, in the culture with 200 g/L of crude glycerol and 20 g/L of CFFA (Table 5), were comparable to the concentrations achieved in the process performed on the low-cost medium with 220 g/L of crude glycerol (Table 4).

Purification of the product from the culture broth obtained after the biosynthesis process might be the second cost-consuming factor of the production process. In the earlier study, we observed that *Y. lipolytica* Wratislavia K1 preferred utilization of by-products of erythritol biosynthesis (i.e., mannitol, arabitol, organic acids) rather than erythritol consumption from the culture broth [7,39]. The unique feature of this strain enables following the idea of cleaner production through the implementation of the natural purification process in which the selectivity of erythritol in the post-culture broth might be increased. In this study, the broth obtained after erythritol production by cultures with *Y. lipolytica* A-6 was collected, separated from biomass, sterilized, supplemented with nutrients, and used as the medium for the Wratislavia K1 bioreactor batch culture. For comparison, the process was conducted at pH 3.5 and 4.5, which are the conditions close to promoting erythritol [16] and biomass production [20], respectively. The comparison of the composition of the initial medium (Figure 2) and the broth after cultivation showed that the selectivity of the erythritol increased from 72% to 97% in the culture performed at pH 3.5. At pH 4.5, its selectivity was slightly lower (96%); nevertheless, the process was significantly shorter, as it lasted only 49 h. It should be mentioned that, on an industrial scale, purification of erythritol from a mixture of polyols is problematic due to their similar chemical structures. Increasing the selectivity of erythritol in the culture broth is therefore beneficial, although further purification steps are required to obtain the final commercial product. After the process performed in this study, the biomass level reached 22 g/L, and its protein content reached 40.4%, meeting the standards recommended for fodder yeast. Worth noticing is that *Y. lipolytica* yeast is non-pathogenic and was recognized as safe (GRAS status) by the Food and Drug Administration (USA) and might be successfully applied for the fodder purposes [40].

## 5. Conclusions

It seems that one of the most important tasks for biotechnology is not so much the possibility of proposing new compounds and products but their production through environmentally-friendly processes. So far, the interest of scientists has focused mainly on the use of industrial waste as a substrate for the biosynthesis of new compounds. It should be remembered, however, that the

proposed process will only be effectively attractive if it does not generate new waste and does not require the use of compounds adverse to the natural environment. In this work, *Y. lipolytica* A-6 was able to produce 108.0 g/L of erythritol with a production rate and yield of 1.04 g/Lh and 0.45 g/g, respectively, only in the two-component low-cost medium containing only crude glycerol and CSL. In turn, the use of *Y. lipolytica* Wratislavia K1 was proven to be effective for the natural purification of the culture broth and resulted in an increase of erythritol selectivity from 72% to 97% and a production of 22.0 g/L of biomass with a high protein content (40.4%). Summarizing, in this study, we have proposed a modern environment-friendly process by *Y. lipolytica* yeast that allows valorization of crude glycerol into erythritol, natural purification of the culture broth, and by-production of biomass suitable for animal feed.

**Author Contributions:** Conceptualization, L.T.-H. and A.R.; methodology, L.T.-H., A.R.; software, L.T.-H.; formal analysis, L.T.-H.; investigation, L.T.-H., A.R.; writing—original draft preparation, L.T.-H., A.R.; writing—review and editing, L.T.-H.; visualization, L.T.-H.; supervision, A.R. and W.R.; project administration, W.R.; funding acquisition, A.R., W.R. and L.T.-H. All authors have read and agreed to the published version of the manuscript.

**Funding:** The research is co-financed under the Leading Research Groups support project from the subsidy increased for the period 2020–2025 in the amount of 2% of the subsidy referred to Art. 387 (3) of the Law of 20 July 2018 on Higher Education and Science, obtained in 2019.

**Conflicts of Interest:** The authors declare no conflict of interest.

## Abbreviations

Following abbreviations were used in the description of the culture media: CFFA—crude fraction of fatty acids, CSL—corn steep liquor, GGM—glycerol based growth medium, FGM—fatty acid crude fraction-based medium, MS-FM—mineral shake-flasks medium with pure glycerol, FPM—fatty acid crude fraction based production medium with pure or crude glycerol, MPM—mineral production medium with pure or crude glycerol, LPM—low cost production medium with pure or crude glycerol, WPM—waste substrate based production medium with crude fraction of fatty acids and pure or crude glycerol, PPM—purification process medium. Other abbreviations: X—biomass, GLY—glycerol, ERY—erythritol, MAN—mannitol, ARA—arabitol, CA—citric acid, KA— $\alpha$ -ketoglutaric acid, Y<sub>ERY</sub>—erythritol production yield, Q<sub>ERY</sub>—volumetric erythritol production rate, q<sub>ERY</sub>—specific erythritol production rate, GK—glycerol kinase, GPDH—glycerol-3-phosphate dehydrogenase, TK—transketolase, ER—erythrose reductase.

## References

1. Moon, H.-J.; Jeya, M.; Kim, I.-W.; Lee, J.-K. Biotechnological production of erythritol and its applications. *Appl. Microbiol. Biotechnol.* **2010**, *86*, 1017–1025. [\[CrossRef\]](#)
2. Regnat, K.; Mach, R.L.; Mach-Aigner, A. Erythritol as sweetener—Wherefrom and whereto? *Appl. Microbiol. Biotechnol.* **2017**, *102*, 587–595. [\[CrossRef\]](#)
3. Carly, F.; Gamboa-Melendez, H.; Vandermies, M.; Damblon, C.; Nicaud, J.M.; Fickers, P. Identification and characterization of EYK1, a key gene for erythritol catabolism in *Yarrowia lipolytica*. *Appl. Microbiol. Biotechnol.* **2017**, *101*, 6587–6596. [\[CrossRef\]](#)
4. Li, L.; Yang, T.; Guo, W.; Ju, X.; Hu, C.; Tang, B.; Fu, J.; Gu, J.; Zhang, H. Construction of an efficient mutant strain of *Trichosporonoides oedocephalis* with HOG1 gene deletion for production of erythritol. *J. Microbiol. Biotechnol.* **2016**, *26*, 700–709. [\[CrossRef\]](#)
5. Mironczuk, A.M.; Furgała, J.; Rakicka-Pustułka, M.; Rymowicz, W. Enhanced production of erythritol by *Yarrowia lipolytica* on glycerol in repeated batch cultures. *J. Ind. Microbiol. Biotechnol.* **2013**, *41*, 57–64. [\[CrossRef\]](#)
6. Rakicka-Pustułka, M.; Biegalska, A.; Rymowicz, W.; Dobrowolski, A.; Mironczuk, A.M. Polyol production from waste materials by genetically modified *Yarrowia lipolytica*. *Bioresour. Technol.* **2017**, *243*, 393–399. [\[CrossRef\]](#) [\[PubMed\]](#)
7. Tomaszewska, L.; Rywińska, A.; Rymowicz, W. High selectivity of erythritol production from glycerol by *Yarrowia lipolytica*. *Biomass-Bioenergy* **2014**, *64*, 309–320. [\[CrossRef\]](#)
8. Guo, J.; Li, J.; Chen, Y.; Guo, X.; Xiao, D. Improving erythritol production of *Aureobasidium pullulans* from xylose by mutagenesis and medium optimization. *Appl. Biochem. Biotechnol.* **2016**, *180*, 717–727. [\[CrossRef\]](#)

9. Rywińska, A.; Marcinkiewicz, M.; Cibis, E.; Rymowicz, W. Optimization of medium composition for erythritol production from glycerol by *Yarrowia lipolytica* using response surface methodology. *Prep. Biochem. Biotechnol.* **2014**, *45*, 515–529. [\[CrossRef\]](#)
10. Yang, L.-B.; Zhan, X.-B.; Zhu, L.; Gao, M.; Lin, C.-C. Optimization of a low-cost hyperosmotic medium and establishing the fermentation kinetics of erythritol production by *Yarrowia lipolytica* from crude glycerol. *Prep. Biochem. Biotechnol.* **2015**, *46*, 376–383. [\[CrossRef\]](#) [\[PubMed\]](#)
11. Jovanović, B.; Mach, R.L.; Mach-Aigner, A. Erythritol production on wheat straw using *Trichoderma reesei*. *AMB Express* **2014**, *4*, 34. [\[CrossRef\]](#)
12. Lee, D.-H.; Lee, Y.-J.; Ryu, Y.; Seo, J.-H. Molecular cloning and biochemical characterization of a novel erythrose reductase from *Candida magnoliae* JH110. *Microb. Cell Factories* **2010**, *9*, 43. [\[CrossRef\]](#)
13. Savergave, L.S.; Gadre, R.V.; Vaidya, B.K.; Narayanan, K. Strain improvement and statistical media optimization for enhanced erythritol production with minimal by-products from *Candida magnoliae* mutant R23. *Biochem. Eng. J.* **2011**, *55*, 92–100. [\[CrossRef\]](#)
14. Tomaszewska, L.; Rywińska, A.; Gładkowski, W. Production of erythritol and mannitol by *Yarrowia lipolytica* yeast in media containing glycerol. *J. Ind. Microbiol. Biotechnol.* **2012**, *39*, 1333–1343. [\[CrossRef\]](#) [\[PubMed\]](#)
15. Rzechonek, D.A.; Dobrowolski, A.; Rymowicz, W.; Mirończuk, A.M. Recent advances in biological production of erythritol. *Crit. Rev. Biotechnol.* **2017**, *38*, 620–633. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Tomaszewska, L.; Rakicka-Pustułka, M.; Rymowicz, W.; Rywińska, A. A comparative study on glycerol metabolism to erythritol and citric acid in *Yarrowia lipolytica* yeast cells. *FEMS Yeast Res.* **2014**, *14*, 966–976. [\[CrossRef\]](#) [\[PubMed\]](#)
17. Rywińska, A.; Juszczak, P.; Wojtatowicz, M.; Robak, M.; Lazar, Z.; Tomaszewska, L.; Rymowicz, W. Glycerol as a promising substrate for *Yarrowia lipolytica* biotechnological applications. *Biomass-Bioenergy* **2013**, *48*, 148–166. [\[CrossRef\]](#)
18. Rakicka-Pustułka, M.; Rukowicz, B.; Rywińska, A.; Lazar, Z.; Rymowicz, W. Technology of efficient continuous erythritol production from glycerol. *J. Clean. Prod.* **2016**, *139*, 905–913. [\[CrossRef\]](#)
19. Juszczak, P.; Tomaszewska, L.; Kita, A.; Rymowicz, W. Biomass production by novel strains of *Yarrowia lipolytica* using raw glycerol, derived from biodiesel production. *Bioresour. Technol.* **2013**, *137*, 124–131. [\[CrossRef\]](#)
20. Papanikolaou, S.; Blanchard, F.; Rondags, E.; Gardeli, C.; Koutinas, A.; Aggelis, G.; Kampisopoulou, E.; Chevalot, I. Production of secondary metabolites through glycerol fermentation under carbon-excess conditions by the yeasts *Yarrowia lipolytica* and *Rhodospiridium toruloides*. *Eur. J. Lipid Sci. Technol.* **2017**, *119*, 1600507. [\[CrossRef\]](#)
21. Xiaoyan, L.; Yu, X.; Lv, J.; Xu, J.; Xia, J.; Wu, Z.; Zhang, T.; Deng, Y. A cost-effective process for the coproduction of erythritol and lipase with *Yarrowia lipolytica* M53 from waste cooking oil. *Food Bioprod. Process.* **2017**, *103*, 86–94. [\[CrossRef\]](#)
22. Liu, X.; Lv, J.; Xu, J.; Xia, J.; He, A.; Zhang, T.; Li, X.; Xu, J. Effects of osmotic pressure and pH on citric acid and erythritol production from waste cooking oil by *Yarrowia lipolytica*. *Eng. Life Sci.* **2018**, *18*, 344–352. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Liu, X.; Yu, X.; Xia, J.; Lv, J.; Xu, J.; Dai, B.; Xu, X.; Xu, J. Erythritol production by *Yarrowia lipolytica* from okara pretreated with the in-house enzyme pools of fungi. *Bioresour. Technol.* **2017**, *244*, 1089–1095. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Liu, X.; Yu, X.; Zhang, T.; Wang, Z.; Xu, J.; Xia, J.; He, A.; Yan, Y.; Xu, J.; Xiaoyan, L.; et al. Novel two-stage solid-state fermentation for erythritol production on okara–buckwheat husk medium. *Bioresour. Technol.* **2018**, *266*, 439–446. [\[CrossRef\]](#)
25. Rakicka-Pustułka, M.; Lazar, Z.; Rywińska, A.; Rymowicz, W. Efficient utilization of inulin and glycerol as fermentation substrates in erythritol and citric acid production using *Yarrowia lipolytica* expressing inulinase. *Chem. Pap.* **2016**, *70*, 1452–1459. [\[CrossRef\]](#)
26. Cybulski, K.; Tomaszewska-Hetman, L.; Rakicka, M.; Juszczak, P.; Rywińska, A. Production of pyruvic acid from glycerol by *Yarrowia lipolytica*. *Folia Microbiol.* **2019**, *64*, 809–820. [\[CrossRef\]](#)
27. Makri, A.; Fakas, S.; Aggelis, G. Metabolic activities of biotechnological interest in *Yarrowia lipolytica* grown on glycerol in repeated batch cultures. *Bioresour. Technol.* **2010**, *101*, 2351–2358. [\[CrossRef\]](#)

28. Tomaszewska-Hetman, L.; Rywińska, A. Erythrose reductase as an enzyme responsible for erythritol overproduction in *Yarrowia lipolytica* yeast grown on glycerol media. *Electron. J. Pol. Agric. Univ.* **2015**, *18*. Available online: <http://www.ejpau.media.pl/volume18/issue4/art-08.html> (accessed on 15 July 2020).
29. Kamzolova, S.V.; Morgunov, I.G. Biosynthesis of pyruvic acid from glycerol-containing substrates and its regulation in the yeast *Yarrowia lipolytica*. *Bioresour. Technol.* **2018**, *266*, 125–133. [[CrossRef](#)]
30. Lazar, Z.; Liu, N.; Stephanopoulos, G. Holistic approaches in lipid production by *Yarrowia lipolytica*. *Trends Biotechnol.* **2018**, *36*, 1157–1170. [[CrossRef](#)]
31. Tomaszewska-Hetman, L.; Rywińska, A. Erythritol biosynthesis from glycerol by *Yarrowia lipolytica* yeast: Effect of osmotic pressure. *Chem. Pap.* **2016**, *70*, 272–283. [[CrossRef](#)]
32. Yang, L.-B.; Zhan, X.-B.; Zheng, Z.-Y.; Wu, J.-R.; Gao, M.-J.; Lin, C.-C. A novel osmotic pressure control fed-batch fermentation strategy for improvement of erythritol production by *Yarrowia lipolytica* from glycerol. *Bioresour. Technol.* **2014**, *151*, 120–127. [[CrossRef](#)] [[PubMed](#)]
33. Liu, X.; Wang, X.-F.; Xu, J.; Xia, J.; Lv, J.; Zhang, T.; Wu, Z.; Deng, Y.; He, J. Citric acid production by *Yarrowia lipolytica* SWJ-1b using corn steep liquor as a source of organic nitrogen and vitamins. *Ind. Crop. Prod.* **2015**, *78*, 154–160. [[CrossRef](#)]
34. Xi, Y.-L.; Chen, K.; Dai, W.-Y.; Ma, J.; Zhang, M.; Jiang, M.; Wei, P.; Ouyang, P. Succinic acid production by *Actinobacillus succinogenes* NJ113 using corn steep liquor powder as nitrogen source. *Bioresour. Technol.* **2013**, *136*, 775–779. [[CrossRef](#)] [[PubMed](#)]
35. Souza, A.F.; Rodríguez, D.M.; Ribeaux, D.R.; Luna, M.A.C.; Silva, T.A.L.; Andrade, R.F.S.; Gusmão, N.B.; Campos-Takaki, G.M.; E Silva, T.L. Waste soybean oil and corn steep liquor as economic substrates for bioemulsifier and biodiesel production by *Candida lipolytica* UCP 0998. *Int. J. Mol. Sci.* **2016**, *17*, 1608. [[CrossRef](#)]
36. Park, E.-H. Role of osmotic and salt stress in the expression of erythrose reductase in *Candida magnoliae*. *J. Microbiol. Biotechnol.* **2011**, *21*, 1064–1068. [[CrossRef](#)]
37. Kona, R.; Qureshi, N.; Pai, J. Production of glucose oxidase using *Aspergillus niger* and corn steep liquor. *Bioresour. Technol.* **2001**, *78*, 123–126. [[CrossRef](#)]
38. Chatzifragkou, A.; Papanikolaou, S. Effect of impurities in biodiesel-derived waste glycerol on the performance and feasibility of biotechnological processes. *Appl. Microbiol. Biotechnol.* **2012**, *95*, 13–27. [[CrossRef](#)]
39. Rzechonek, D.A.; Neuvéglise, C.; Devillers, H.; Rymowicz, W.; Mironczuk, A.M. EUF1—A newly identified gene involved in erythritol utilization in *Yarrowia lipolytica*. *Sci. Rep.* **2017**, *7*, 12507. [[CrossRef](#)]
40. Coelho, M.A.Z.; Amaral, P.F.F.; Belo, I. *Yarrowia Lipolytica*: An industrial workhorse. In *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*; Mendez-Vilas, A., Ed.; Formatex Research Center: Badajoz, Spain, 2010; pp. 930–944.

